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In vivo stability and kinetics of absorption and disposition of 3′ phosphopropyl amine oligonucleotides

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ABSTRACT

Development of oligonucleotide derivatives as therapeutic agents requires an understanding of their pharmacokinetic behavior. The *in vivo* disposition and stability of a prototype of such compounds are reported here. The compound studied, a relatively G-rich 38 base 3′ phosphopropyl amine oligonucleotide (TFO-1), was cleared from the circulation with a half-life of approximately 10 minutes, displaying distribution kinetics consistent with a two compartment model. TFO-1 was also readily absorbed into circulation from the peritoneal cavity. All tissues examined except brain accumulated the compound reaching concentrations calculated to be in the micromolar range. TFO-1 was found to be stable in circulation and in tissues in that a large fraction of intact material was detected 8 hours after injection, as assessed by gel electrophoresis. Approximately 20–30% of the injected dose was excreted in the urine over an 8 hour period. These results suggest that G-rich oligonucleotides, minimally modified at the 3′ end, are relatively stable *in vivo* and have distribution kinetics favorable to use as therapeutic agents.

INTRODUCTION

Oligonucleotides and their derivatives have been shown to specifically inhibit gene expression. Because of their potential to control diseases of known genetic etiology, development of these compounds as therapeutic agents is of great interest. The use of antisense oligonucleotides to inhibit gene expression by means of duplex formation with mRNA has been recently reviewed (1,2). The triple helix approach is based on the ability of single stranded DNA to recognize and specifically bind to double stranded DNA sequence motifs. Triple helix formation results from Hoogsteen or reverse Hoogsteen hydrogen bonding between a duplex pair and an oligonucleotide base (1–4). Such complexes were first demonstrated using homopolymers and simple copolymers (5–8). In general, these were designed as a polypyrimidine strand in parallel orientation to a polypurine segment within duplex DNA. These complexes, however, become relatively weak at physiological pH. More recently, it has been shown that triple helix-forming oligonucleotides (TFO's) based on formation of G-G:C and T-A:T triplets have the ability to bind stably and with high selectivity to target DNA at physiological pH and ion concentrations (9–13). Inhibitory effects of TFO's on protein-DNA interactions (9,10,14–16) and specific gene transcription (9,14) have been demonstrated in cell free systems. Inhibition of gene expression has also been recently shown in intact cultured cells (10,11,16).

In the extracellular concentration range of 0.1 to 7 μM, oligonucleotides readily enter cultured cells (10,17–22), reaching intracellular (10,11,18) and nuclear (10,11,23) concentrations which equal or exceed that of the extracellular medium within 2–4 hours. Hence, these compounds may be viable therapeutic agents if their *in vivo* distribution and stability characteristics are favorable.

In cultured cells, culture media and human serum, degradation of unmodified oligodeoxynucleotides appears to take place primarily as a result of 3′ exonuclease activity (24–26). We (unpublished results) and others (10,24–26) have found that modifications to the 3′ end of oligonucleotides greatly enhance their stability to exonuclease degradation in serum and cultured cells. In our hands, addition of a phosphopropyl amine group to the 3′ end of the oligonucleotide during synthesis (27,28) is an effective modification in this regard.

Little information is available concerning the *in vivo* disposition and stability of oligonucleotides. Emlen and Mannik (29) observed serum levels and organ distribution of unmodified single and double stranded DNA (ssDNA and dsDNA) following intravenous (IV) injection into mice and concluded that ssDNA was cleared rapidly from the circulation and distributed to the tissues with a large portion of the injected dose going to the liver. Chen et al. (30) have described the plasma half-life and distribution profile of a 12 base deoxyoligonucleoside methylphosphonate injected into mice. They found that the compound was eliminated from the plasma with a half-life of 6 minutes and that about 70% of the injected dose was excreted

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in the urine within 2 hours. Agrawal et al. (31) have recently reported the biodistribution of a 20 base oligodeoxynucleotide phosphorothioate in mice.

In an effort to understand the pharmacokinetics of minimally modified TFO's, we present the following study in which we describe the absorption, distribution and metabolism of a G-rich 38 base oligodeoxynucleotide containing internucleotidyl phosphodiester linkages and a phosphopropyl amine group at the 3′ end. We demonstrate that this compound, with only a 3′ end modification, is efficiently accumulated by tissues and remains intact for at least 8 hours *in vivo*.

MATERIALS AND METHODS

Preparation of compounds

Oligonucleotides for preparation of internally labeled oligonucleotide were synthesized on an automated MilliGen 8700 DNA synthesizer (Millipore Corp., Milford, MA). Unlabeled oligonucleotide was synthesized on a MilliGen 8800 Large Scale DNA synthesizer. All precursors were standard β-cyanoethyl phosphoramidites purchased from Millipore. Amino-modifier CPG purchased from Glen Research (Sterling, VA) was used as starting material support in order to introduce a phosphopropyl amine group at the 3′ end of the oligonucleotides (27,28), thus preventing degradation by nucleases.

For large-scale preparations, the deprotected compound was purified by anion-exchange chromatography (Q-Sepharose HP; Pharmacia/LKB; Piscaway, NJ). The eluted full length compound was concentrated and desalted on an Amicon (Danvers, MA) ultrafiltration device using a UM1 membrane with a 3,000 MW cutoff.

Unlabeled preparations of TFO-1 were characterized by ultraviolet light spectrophotometry, reversed phase HPLC and NMR (400 MHz). In addition, oligonucleotides were 5′ end-labeled with γ-^{32}P ATP (DuPont, Wilmington, DE) using polynucleotide kinase (Boehringer-Mannheim, Indianapolis, IN) and analyzed by polyacrylamide gel electrophoresis (PAGE) (12% acrylamide gel, in 80 mM tris, 2 mM EDTA, 90 mM boric acid (TEB), 7 M urea). The results of these analyses were consistent with the structure given in Figure 1D. The molecular weight of TFO-1 was calculated to be 12,374.

Internally ^{32}P-labeled TFO-1 was prepared as two fragments (See Figure 1). A 23 base fragment bearing the 3′ amine modification was 5′ end-labeled with γ-^{32}P ATP using polynucleotide kinase. The other fragment was 15 bases long. The two fragments were annealed to a complementary oligonucleotide. The 5′ ^{32}P-labeled fragment was ligated to the 3′ end of the 15-mer using T4 ligase (Boehringer-Mannheim) and gel purified using PAGE (12% acrylamide gel, TEB, 7 M urea). The purified oligonucleotide was then desalted on a NAP-10 column (Pharmacia/LKB) equilibrated with phosphate buffered saline (PBS). The ^{32}P labeled material was adjusted to a specific activity of 400,000 cpm/mg and a concentration of 13.0 mg/ml.

Animal Injection and Tissue Collection

Two to four month old female BALB/cAnNhsd mice (Harlan-Sprague Dawley, Inc., Indianapolis, IN) weighing approximately 20 grams each were injected with internally ^{32}P-labeled or unlabeled TFO-1 at 130 mg/kg in 0.2 ml PBS intraperitoneally (IP) or IV via the lateral tail vein.

At various time points after injection, mice were anesthetized by Metofane inhalation and blood was collected via cardiac puncture (25 G needle, 1cc syringe, coated with a solution of sodium heparin, 10,000 units/ml) and placed on ice. Mice were then sacrificed by cervical dislocation and the liver, spleen, brain, kidneys, heart, lung, external ear (used as a source of skin) and a portion of the hind limb muscle were removed, rinsed in ice-cold PBS, immediately frozen in liquid nitrogen and stored at −70°C until use. Animals injected IV were sacrificed 3, 10, 20, 40, 120 and 240 minutes after injection. Those injected IP were sacrificed at 3, 30, 120, 240 and 480 minutes.

Absorption and Distribution Studies

An aliquot of whole blood (100 μl) was added to 1 ml of Soluene (Packard Instrument Co., Meriden CT) and the remaining whole blood was spun at 10,000×g in an Eppendorf 5414 centrifuge for 5 minutes. Plasma was collected and 100 μl was added to 1 ml of Soluene. Frozen tissues were weighed and a portion of each was solubilized individually in 1 ml of Soluene and incubated at 37°C for 15 hours. H_2O_2 was added to decolorize the tissue samples. Radioactivity in the samples was measured using a Beckman LS 3801 Scintillation Counter (Beckman Instrument Co., Fullerton, CA).

Tissue samples from uninjected animals were solubilized as above and counts from these samples were subtracted from the values of the corresponding samples from injected animals to correct for the small amount of chemiluminescence resulting from tissue solubilization.

For some studies, the amount of tissue-associated compound was expressed as the 'percent of injected dose', calculated as follows: (Mass of compound found in the tissue sample ÷ Mass of the tissue sample)×(Total mass of the tissue) ÷ (Total mass of compound injected)×(100%). For muscle, the 'Total mass of the tissue' was considered to be the mass of all skeletal muscle, not just that of the leg muscle used.

In Vivo Oligonucleotide Stability Studies

Ten μl of plasma was incubated at 65°C for 10 minutes and 5′ end-labeled with γ-^{32}P ATP using polynucleotide kinase and then extracted with an equal volume of phenol:chloroform (1:1). The aqueous layer was removed, dried in a Savant Speed-Vac concentrator (Savant Instruments, Inc, Farmingdale, NY), and soaked in 400 μL of 85% ethanol for 3 hours at room temperature to remove salts and unincorporated ^{32}P. The precipitate was recovered by centrifugation (15,000×g, 5 min), dissolved in H_2O, denatured using 50% formamide at 65°C and subjected to PAGE (12% acrylamide gel, TEB, 7M urea). Under these conditions, there was no selective loss of oligonucleotide fragments of 10 bases or greater.

For tissue samples, approximately 50 mg of each sample was homogenized using a Dounce tissue grinder in 1 ml of buffer containing 80 mM tris, 5 mM EDTA, and 1% SDS. The homogenate was aspirated through an 18G needle with a 1cc syringe and was then extracted with phenol:chloroform (1:1). An aliquot of the aqueous layer representing approximately 5 mg of tissue was treated with RNase A (10 mg/ml) at 37°C for 15 hours. The samples were then incubated at 60°C for 1 hour with proteinase K (Boehringer-Mannheim) at 4 mg/ml and 0.1% SDS. Following incubation, samples were phenol and chloroform extracted, dried and soaked in 400 μL of 85% ethanol for 3 hours at room temperature. The precipitate was recovered by centrifugation, dissolved in H_2O and 5′-end-labeled with γ-^{32}P

ATP using polynucleotide kinase. The samples were denatured using 50% formamide at 65°C and subjected to PAGE (12% acrylamide gel, TEB, 7 M urea). Following electrophoresis the gels were dried and exposed to X-ray film.

Excretion Studies

Mice were injected IV or IP with 130 mg/kg of internally labeled oligonucleotide and placed in metabolism cages until they were sacrificed at 30 minutes and 4 hours in IV studies and 30 minutes, 4 hours and 8 hours in IP studies. The urine deposited in the cage was pooled with that collected at sacrifice for each animal and a portion was analyzed by liquid scintillation counting (LSC). To assess the physical state of the compound in the urine the collected urine was end-labeled and analyzed by PAGE and autoradiography, as described for plasma samples.

RESULTS

Absorption and distribution studies

Oligonucleotide, TFO-1, internally labeled with ^{32}P, was injected either IV or IP as described in Materials and Methods. Blood was removed by cardiac puncture at several time points and the amount of oligonucleotide present in the plasma was assessed by LSC. In IV studies, blood was collected six times over a 4 hour period. Due to slower absorption, IP studies were conducted over a 6 hour period. Figure 2 shows that, following IV injection, oligonucleotide was rapidly cleared from the blood with a half-life of less than 10 minutes. The non-linear curve suggests a two compartment model for distribution (32). As expected, oligonucleotide clearance was delayed and peak plasma concentration of oligonucleotide was lower when given IP. Plasma levels of oligonucleotide reach a maximum about 30 minutes after IP injection and appear to remain relatively constant for approximately 90 minutes, declining slowly thereafter. The difference in distribution kinetics following IV and IP injection suggests that, in the case of IP injection, absorption from the peritoneal cavity was the limiting factor in distribution. Area under the plasma concentration vs. time curve to 240 minutes (AUC), was calculated to be 27 mg·min/mL for the IV case and 30 mg·min/mL for the IP case.

In order to determine the uptake of compound into tissues, oligonucleotide concentration was also measured over a 4 hour course following IV injection and over 8 hours following IP injection. Seventy to 100 mg of brain, heart, kidney, liver, lung, muscle, spleen and external ear was harvested, solubilized and prepared for LSC as described in Materials and Methods. The absorption profiles, presented in Figure 3, Panel A, suggest that immediately after IV injection, the oligonucleotide concentration in kidney followed that of the plasma, as it does in lung, ear, heart and brain. That is, the peak concentrations measured in these tissues occurred at 3 minutes, the earliest time points taken (See also Table 1). The concentration in these tissues stabilized and remained constant through at least 4 hours while plasma levels diminished to near zero. In these tissues, a portion of the oligonucleotide present at the early time points ($\leq$20 minutes) may be contributed by the residual blood left in the tissue after harvest, whereas, following IV injection, accumulation at times greater than 30 minutes is not affected by blood-borne oligonucleotide, which is then too dilute to be measured.

Liver continued to accumulate oligonucleotide for 2 hours after injection and thereafter remained relatively constant through the 4 hour measurement. Oligonucleotide concentration in muscle remained fairly constant during the course of the experiment, reaching peak values within 10 minutes after injection. The concentration in spleen initially declined, coinciding with the decrease measured in plasma, then slowly increased, reaching a steady state between 2 and 4 hours. These data clearly indicate that TFO-1 is accumulating in all the tissues measured with the exception of brain.

Following IP injection (Figure 3B), kidney, lung, ear, heart, and brain concentration also followed the IP plasma profile seen in Figure 2B, whereas the concentration profiles of muscle and liver followed essentially the same profile as seen after IV injection. Again, by 4 hours after IP injection, all tissues have reached steady state concentrations while plasma levels continue to decline toward zero.

The maximum oligonucleotide concentrations reached in tissues

A) 5'-TGGGTGGGGTGGGGT-OH-3'
B) 5'-GGGGGGGTGTGGGGTGTGGGGTG-NH_2-3'
C) 3'-NH_2-CACCCCACCCCACCCCCCCACACC-5'

D) 5'-TGGGTGGGGTGGGGTGGGGGGGTGTGGGGTGTGGGGTG-NH_2 -3'

Figure 1. Internal labeling of TFO-1. Oligos A,B, & C were synthesized by standard methods and gel purified. Oligo B was 5' end-labeled using γ ^{32}P ATP and polynucleotide kinase. Oligos A & B were annealed with oligo C and ligated with T4 ligase. The labeled, ligated oligo D was then gel purified. NH_2 indicates the presence of a phosphopropyl amine group (NH_2-CH_2-CH(OH)-CH_2-O-PO_2-O-) at the 3' end. The underlined G (G) indicates the base labeled at the 5' end. The molecular weight of oligo D (TFO-1) was calculated to be 12,374.

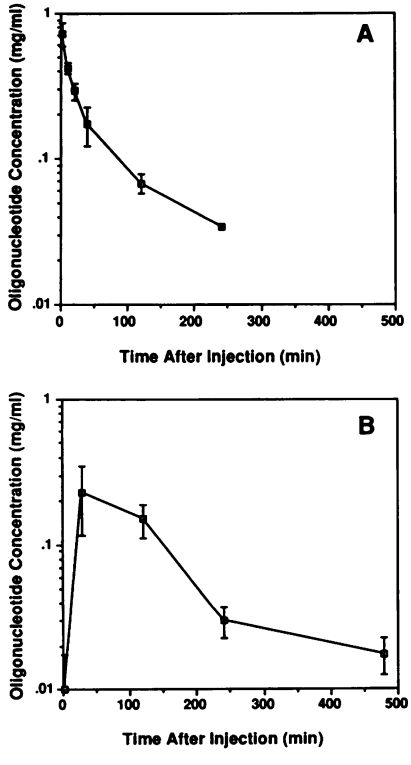

Figure 2. Plasma concentration vs. time profiles following 130 mg/kg dose of internally ^{32}P labeled TFO-1 injected IV (panel A) and IP (panel B). Data points represent the mean ± standard deviation of 3 to 5 animals.

following IV and IP injection are shown in Table 1. Consistent with the plasma concentration (Figure 2), the time required to reach maximum concentration after IP injection was, in general, longer than that following IV injection. Only brain and muscle reached maximum levels at 3 minutes. The apparent oligonucleotide concentration measured in brain is so low that it may reflect oligonucleotide in residual blood in the tissue during processing and may not be associated with the tissue. Muscle tissue from mice injected IP showed a slow but non-significant increase over the course of the experiment (26.7 mg/kg after 10 minutes and 29.3 mg/kg after 4 hours). No significant changes occurred in tissue associated oligonucleotide concentration between 4 hours and 8 hours after IP injection.

The tissue concentrations measured during the steady state are compared for IV and IP administration in Table 1. Brain, ear, muscle, spleen and heart levels were found to be identical, however, kidney and liver concentrations were somewhat higher after IP injection. This difference may be a result of the longer duration of elevated plasma levels observed after IP injection.

The effect of dose on plasma and tissue levels was examined in an experiment in which animals were injected IP with a 37 base oligonucleotide (TFO-2) of similar composition to that shown in Figure 1, but with a different nucleotide sequence. In this experiment, mice were injected with either 19 mg/kg or 130 mg/kg and plasma and tissue levels of oligonucleotide were measured after 3 hours. The data, displayed in Table 3, show that liver, kidney and muscle contained approximately equal percentages of the injected dose whether they were exposed to a dose of 19 mg/kg or 130 mg/kg. In plasma, on the other hand, the ratio of dose percentages was 10.9, greater than the ratio of the doses administered (6.8). These data suggest that the clearance rate from plasma is slower at higher injected doses.

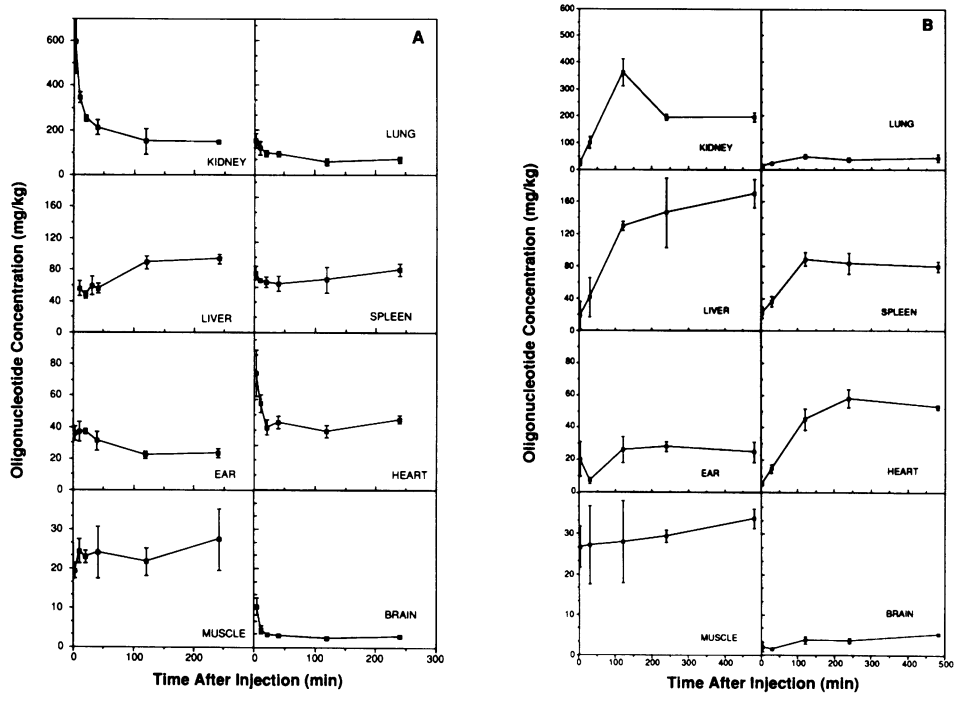

Figure 3. Tissue concentration vs. time profiles of 130 mg/kg dose of internally ^{32}P labeled TFO-1 injected IV (panel A) or IP (panel B). Data points represent the mean ± standard deviation of 3 to 5 animals. Note that 4 different scales have been used on the y-axes and that the x-axes of panel A range from 0 to 300 minutes while the those of panel B range from 0 to 500 minutes.

Table 1. Accumulation of Oligonucleotide in Tissues Following Intravenous and Intraperitoneal Injection

	Maximum Concetration (mg/kg)		Time to Maximum Concentration (min)		Concentration 4 Hours 4 Hours After Injection (mg/kg)		% of Injected Dose 4 Hours After Injection	
	IV	IP	IV	IP	IV	IP	IV	IP
Kidney	597.6	361.6	3	120	146.2	193.2	1.5	2.1
Liver	93.4	169.5	120	120	93.4	145.9	3.9	5.9
Heart	74.6	57.5	3	120	45.1	57.5	0.2	0.2
Lung	151.3	48.8	3	120	37.0	67.0	0.5	0.3
Spleen	79.4	88.8	3	120	79.4	83.7	0.3	0.4
Muscle	27.4	33.8	10	3	27.4	29.3	4.2	5.2
Ear	37.1	27.9	3	3	23.3	27.9	0.1	0.1
Brain	10.3	5.0	3	480	2.6	3.4	0.1	0.1

[1] Percent of injected dose was calculated as described in Materials and Methods.

In Vivo Stability Studies

In order to determine the physical state of the injected oligonucleotide, unlabeled compound was injected IV or IP at a 130 mg/kg dose. The animals were sacrificed at various times as described in Materials and Methods and the oligonucleotide present in plasma and tissues was extracted, end-labeled with ^{32}P and subjected to PAGE. Figure 4 shows an autoradiogram resulting from analysis of labeled plasma fractions. Oligonucleotide remained largely intact in the plasma over the course of the 8 hour experiment, although some degraded oligonucleotide was detected in the 37 to 27 base range. Analysis of plasma from PBS injected control animals revealed labeled material below 26 bases indicating that the bands observed below 26 bases result from endogenous nucleic acids in plasma itself rather than injected oligonucleotide. The 77 base band results from an oligonucleotide that was added to the kinase reaction mixtures at a concentration of 1 mM as a standard to indicate the approximate concentration of the injected oligonucleotide in the sample. Comparison of this standard to other lanes suggests that the concentration of intact TFO-1 in the plasma 8 hours after IP injection was 1−2 μM which, for this compound, is equivalent to 12−24 mg/ml. Although at early time points the exposure has exceeded the linear range of the film, the autoradiography at 8 hours is consistent with the LSC data derived from measurement of internally labeled oligonucleotide shown in Table 1.

In similar experiments, liver samples harvested after IP injection were end-labeled and analyzed by PAGE and autoradiography. Results, shown in Figure 5, demonstrate that the oligonucleotide associated with the liver also remained intact over the 8 hour course of the experiment. As shown by the bands in the lane marked CTR, the heavy, low molecular weight bands in all lanes are contributed by phosphorylated endogenous nucleic acids (perhaps degraded mRNA) and not the injected oligonucleotide. Although some degradation bands can be seen after 120 minutes the great majority of the TFO-1 remains intact even after 8 hours *in vivo*. Comparison of the full-length TFO-1 bands (indicated by the arrow) with 1 μM external standard band shows that the concentration of full length TFO-1 in the liver samples is about 1 μM (12 mg/mL) at 3 minutes and about 10 μM (120 mg/mL) after 4 hours, again consistent with the LSC analysis.

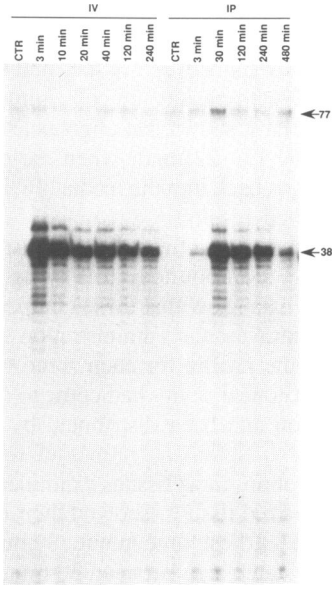

Figure 4. PAGE/autoradiography analysis of TFO-1 from plasma of animals injected IV or IP with a dose of 130 mg/kg. Samples were harvested at the times indicated at the top of each lane. The band at 77 bases represents a standard added to the kinase reaction at the concentration of 1 μM. The 38 base band indicates the mobility of TFO-1. Lanes marked CTR are samples from animals injected with PBS. These are included to demonstrate the background contributed by plasma. The plasma samples were extracted and ^{32}P end-labeled as described in Materials and Methods.

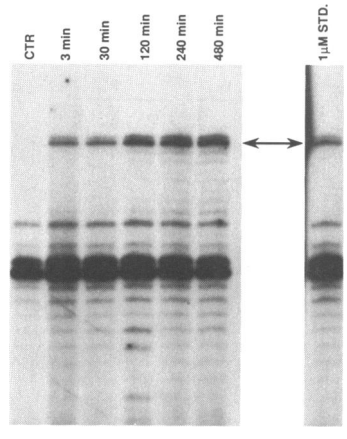

Figure 5. PAGE/autoradiography analysis of nucleic acids from liver samples of animals injected IP with a dose of 130 mg/kg. The samples were harvested after injection at the times indicated at the top of each lane, extracted and end-labeled with ^{32}P as described in Materials and Methods. CTR indicates a sample from an animal injected with PBS showing the bands caused by endogenous nucleic acids. 1 μM STD shows a sample from a PBS-injected animal that was spiked with TFO-1 at a concentration of 1 μM. The arrow indicates the mobility of TFO-1.

Table 2. Effect of Dose on Distribution of Oligonucleotide 3 Hours after Intraperitoneal injection.

	Percent of Injected Doses[1]			
Dose (mg/kg)	Plasma	Liver	Kidney	Muscle
19	0.56	4.88	1.75	3.39
130	6.10	5.15	2.44	2.68
Ratio (130/19)	10.89	1.06	1.39	0.79

[1]Percent of injected dose was calculated as described in Materials and Methods.

Table 3. Cumulative urinary excretion of HIV38p following injection of 130 mg/kg.

	Percent of Injected Doses[1]		
Route of Injection	30 min.	4 hr.	8 hr.
IV	21.9 ± 2.14	24.9 ± 4.03	ND
IP	3.1 ± 0.14	19.0 ± 1.64	30.4

[1] Values shown are the mean and standard deviation of 3 to 6 animals. The 8 hour, IP value represents a measuement of urine pooled from 4 animals. Percent of injected dose was calculated as described in Materials and Methods.

Similar analysis was performed on samples of all tissues harvested (data not shown). Results of these experiments were quite similar to those of the liver analysis depicted in Figure 5. Results of these stability studies demonstrate that the internally labeled oligonucleotide, measured in earlier experiments and described in Table 1, are largely intact even after 8 hours in the animals.

Excretion studies

Considering the high concentration of oligonucleotide observed in the kidney and its rapid disappearance from the plasma, it was of interest to examine the urine of mice injected with oligonucleotide to determine the kinetics of urinary excretion. Urine was collected from mice 30 minutes and 4 hours after IV injection and 30 minutes, 4 hours and 8 hours after IP injection. Table 3 shows that following IV injection, 21.9% of the injected dose is excreted into the urine during the first 30 minutes after injection. This value rises only slightly to 24.9% after 4 hours. As might be expected from the low plasma levels, only a small percentage of the injected dose is found in the urine 30 minutes after IP injection. This value rises to 19% by 4 hours and to 30% at 8 hours.

In order to assess the physical state of the excreted oligonucleotide, a portion of the collected urine was used as substrate in a polynucleotide kinase reaction and the labeled material was subjected to PAGE and autoradiography. Figure 6 shows that, while some degradation has occurred, much of the oligonucleotide in the urine is high molecular weight; i.e., 38 to 26 bases in length. These data indicate that intact oligonucleotide was excreted in the urine but that the ≥70% of total injected dose is not excreted within 8 hours after injection.

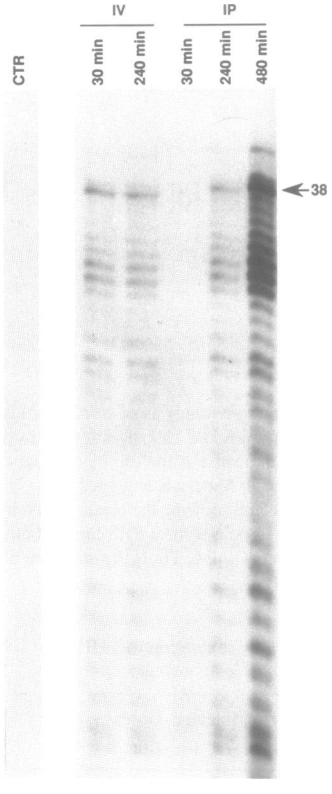

Figure 6. PAGE/autoradiography analysis of urine collected from animals injected IV or IP with 130 mg/kg and end-labeled with ^{32}P as described in Materials and Methods. Samples were collected at the times indicated at the top of each lane. CTR is a urine sample from an animal injected with PBS showing endogenous background. The arrow indicates migration of full length TFO-1.

DISCUSSION

We have described the distribution and metabolism of a 38 base oligonucleotide modified at the 3′ end by addition of phosphopropyl amine. The information reported here, we believe, should be valid not only for the particular sequence shown in Figure 1, but also for other oligodeoxynucleotides of the same general size, base composition and end structure.

The present study reveals that these compounds are rapidly cleared from circulation with a $T_{1/2}$ of about 10 minutes. These findings are consistent with other reports demonstrating rapid plasma depletion of large ssDNA (29,33,34), and methylphosphonate (30) and phosphorothioate (31) oligodeoxynucleotide. The plasma concentration vs. time profile following IV injection is consistent with a two compartment model (32) and similar to that reported by Chen. Such a kinetic model suggests that the compound in the plasma is in equilibrium with that in some other compartment, presumably one or more tissues. That an equilibrium exists between tissues and the extracellular milieu is consistent with results of cell culture experiments. Several investigators have shown that oligonucleotides are readily taken up by cells (10,17−22) and, when the culture media is changed, release oligos back into the media (18,19, Marshall and Zendegui, unpublished results).

Our IP injection study is the first report of distribution kinetics of oligonucleotide compounds following extravascular administration. The data show that these compounds are readily absorbed from the extravascular administration site. Comparison of the plasma vs. time profile for each route suggests that the compound in the peritoneal cavity had come to equilibrium with that in the circulation and been distributed by the 240 minute measurement.

Following IV injection, most tissues examined, especially the more highly vascularized tissues, showed their highest apparent concentration at the earliest time point, 3 minutes, and then declined to a steady state value between 30 minutes and 2 hours after injection. Since the tissues were rinsed but not perfused after harvesting, these early values may reflect the high oligonucleotide concentration in the residual blood left in the tissue. In brain, the very low values at 4 hours may reflect residual blood in the tissue and/or chemiluminescence measurement error and not true absorption into brain tissue. In any case, the data suggest that, at least in healthy animals, these compounds do not cross the blood-brain barrier with great efficiency, if at all.

It is clear from the steady state levels reached after either IV or IP injection (Figure 3 & Table 1) that a large portion of the TFO-1 has been transferred from the plasma to the tissue compartment. It is unclear from this study what portion of the tissue-associated compound is actually intracellular and what portion might be interstitial. However, extrapolation from reports of cellular uptake experiments performed in cultured cells (10,11,17−23) suggests that much of the compound is intracellular. Further experiments will be required to clarify this point.

The rank order of concentrations of oligonucleotide 4 hours after either IV or IP injection was kidney > liver > spleen > heart, lung > muscle, ear >> brain (see Table 1). Chen et al.

(30) also found kidney to have the highest concentration; however, in comparison to the data presented here, liver and spleen were lower while lung was higher. These discrepancies are probably due either to differences in length (12 bases vs. 38 bases) or the uncharged nature of the oligonucleotides used in Chen's study. It would not be surprising that either length or backbone structure would influence distribution of these compounds.

The steady state concentrations for all tissues from IP injected animals measured at 4 hours were somewhat higher than from those of the IV treated group. Liver concentration was 56% higher in the IP treated group and kidney was 43% higher. These differences may result from the longer duration of elevated plasma concentrations seen in IP injected animals (see Figure 2). That is, the higher plasma concentration between 30 minutes and 2 hours seen following IP injection may shift the plasma-tissue equilibrium of the compound resulting in higher tissue levels. The relationship between dose and plasma concentration and between plasma and tissue concentrations was not examined in these studies, and whether tissue absorption follows strict first order kinetics is not known. The results of the experiment shown in Table 2 suggests that absorption and/or distribution following IP injection may not be a first order process. The data show that the ratio of the plasma concentrations (10.8) 180 minutes after administration of 130 or 19 mg/kg doses were greater than the ratio of the doses (6.8) whereas the ratio of the tissue concentrations was close to 1, suggesting that the tissue concentration may not be following the plasma concentration. A detailed study will be needed to understand these relationships. It is clear from this data, however, that the plasma clearance rate is slower at the higher dose than at the lower.

The apparent oligonucleotide concentration of most tissues decreased during the first 1−2 hours after IV injection. Thereafter tissue concentrations reached a plateau, and did not decline significantly over the remainder of the experiment. Following either IP or IV injection, the liver appeared to continue to accumulate the compound even at the latest times measured. In other experiments, the liver concentration of oligonucleotide remained stable for 16 hours after IP injection of a dose of 19 mg/kg (data not shown). This profile is not consistent with that of methylphosphonates in that Chen reported a 60% decline in the liver concentration between 5 and 120 minutes after injection (30). In isolated organ experiments, single stranded DNA in the range of 75−150 bases was reported to associate avidly with liver whereas small size (5−15 bases) did not (29). The compound used by Chen et al. was 12 bases in length and may have a lower avidity for the liver for that reason. Alternatively, the differences between the Chen et al. data and ours may relate to the uncharged nature of the methylphosphonate backbone.

While it has been demonstrated that modification to the 3′ end of otherwise unmodified oligonucleotides prevents or greatly slows degradation in serum and cultured cells (10,24−26), no information is available regarding the stability of these compounds *in vivo*. The *in vivo* fate of unmodified ssDNA was addressed by Emlen and Mannik (29,33,34). They were unable to demonstrate the presence of intact DNA in the plasma of mice, regardless of size or strandedness, 40 minutes after IV injection of a dose of 0.4 mg/kg. In the studies presented here, we were able to recover intact compound from the plasma 8 hours after injection. Likewise, in tissues, intact compound was present at the latest times examined. Figure 5 shows that most of the liver associated compound has not been degraded even 8 hours after injection. Although we did not directly compare the *in vivo* stability of unmodified oligonucleotides with that of 3′ phosphopropyl amine compounds, it seems clear from our results that 3′ exonucleases are largely responsible for degradation of oligonucleotides *in vivo* as well as in serum and cell extracts. The minor degradation seen in plasma and tissues may result from 3′ exonucleases that are capable of attacking the oligonucleotide, albeit at a very slow rate, in spite of the 3′ cap, or may be due to endonuclease activity. In any case, the fact that tissue-associated compound does not appear to be in decline after 8 hours (Figure 3) and that the material remaining in the tissues is largely intact (Figure 5) indicates that stable tissue concentrations in a therapeutic range may be achievable with 3′ modified oligonucleotides.

A portion of the IV injected dose, 20%, was rapidly excreted in the urine. Although we attempted to measure urine concentrations 3 and 10 minute after IV injection we were unable to obtain any urine from the animals. One would expect that, at some size, oligonucleotides would be too large to be filtered through the renal glomeruli. Our findings suggest that, at 38 bases, these compounds are freely filtered and not reabsorbed. Chen (30) has shown that, after injection of a 12 base methylphosphonate oligonucleotide, 70% of the compound was excreted in the urine. Agrawal (31) reported that approximately 24−30% of a 20 base phosphorothioate compound was excreted in the urine within 12 hours after injection. It seems likely that these discrepancies are attributable to the difference in size but may also have to do with the uncharged methylphosphonate backbone. If the degree of degradation observed in urine is compared to that in plasma (Figure 4), it is clear that a larger portion of the compound is degraded in urine. This may reflect a more rapid filtration rate for smaller oligonucleotide species, or may result from degradation during filtration or storage in the bladder. Further study will be required to reveal the relationship between oligonucleotide size and excretion rate.

It is clear that the amount of accumulation in the tissues sampled is consistent with the 20−30% excreted in the urine. That is, the 95% decrease in plasma concentrations over the four hours following IV injection is not due to urinary excretion. We did not measure biliary excretion in these studies, however, it is clear from the amount of compound absorbed into tissues that if biliary excretion is occurring, it is not rapid.

We have directly accounted for approximately 40−45% of the injected TFO-1: 11−14% in the tissues examined (see Table 1) and about 30% in the urine (see Table 3). The 55−60% unaccounted for could likely be found in bone marrow and blood cells, and in gastrointestinal, vascular and other tissues, which were not tested in this study.

The oligonucleotides used in this study had a very high G content (76%). We have not studied the effects of base composition on the *in vivo* stability or distribution and published studies are difficult to compare due to the extensively modified oligonucleotides used (30,31). It is quite possible that base composition may influence biodistribution and *in vivo* stability of these compounds.

CONCLUSION

The present study shows that G-rich 3′ phosphopropyl amine oligonucleotides remain stable in the circulation, and that following injection they are rapidly cleared from circulation into tissues and urine. The compounds are readily absorbed into tissues

where they appear to be stable for at least 8 hours. The two compartment model suggested by the plasma concentration vs. time curves (Figure 2) would predict that the intact compounds are in equilibrium between the circulation (central compartment) and a second compartment, presumably one or more tissues. The slow elimination from the plasma and the relatively stable levels in the tissues suggest that the tissues to plasma component of the equilibrium is rather slow. In a broader sense, this study also demonstrates that, as a class of compounds, oligodeoxynucleotides containing internucleotidyl phosphodiester linkages and a minimal 3′ modification have favorable absorption and distribution kinetics and sufficient *in vivo* stability to be used as therapeutic agents.

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